NF-κB independent Cell Survival Regulation in Differentiated Skeletal Muscle

Senior Thesis

By

Eric Michael Hill

Undergraduate Program in Biomedical Science

The Ohio State University

2010

Thesis Committee:

Denis C. Guttridge, Advisor

Jill A. Rafael-Fortney

Maureen Geraghty

ABSTRACT

The transcription factor NF-κB has been shown to inhibit the differentiation of skeletal muscle cells via numerous molecular mechanisms [1-4] and is believed to play an integral role in several skeletal myopathies [3, 5]. NF-κB has also been shown to play a vital role in inhibiting programmed cell death by up-regulating specific anti-apoptotic genes following a stressful stimulus [6-8]. Interestingly, it has been reported that terminally differentiated skeletal muscle cells devoid of NF-κB activity do not undergo apoptosis when treated with the inflammatory cytokines interferon-γ and tumor necrosis factor alpha (TNFα) [3]. In this study, we examine the reported phenomenon using TNFα, an extrinsic signal for apoptosis as well as a potent activator of NF-κB, to better determine the role of NF-κB in the survival of skeletal muscle cells at different stages of the differentiation program. Using a C2C12 cell line stably expressing a plasmid coding for a dominant negative form of IκBα (IκBα-SR), it was shown that myoblasts lacking competent NF-κB activity were sensitized to TNFα-induced apoptosis, while identical IκBα-SR myotubes were not. To determine if C2C12 myotubes possess an intact TNFα signaling pathway, Western blotting for phosphorylated forms of p38 and JNK as well as reverse transcriptase-PCR for TNFα-activated genes were performed. It has recently been claimed that skeletal muscle differentiation evokes a natural reduction of apoptotic peptidase activating factor-1 (Apaf-1), a vital pro-apoptotic protein [9]. Western blot analysis of IκBα-SR myoblasts and myotubes confirm that the natural reduction of Apaf-1 is present in C2C12 cultures, as well as indicate that the reduction is not dependent on

NF-κB. To determine if this reduction in Apaf-1 could be the mechanism responsible for the NF-κB independent inhibition of apoptosis seen in skeletal myotubes, C2C12-IκBα-SR myoblasts were transiently transfected with an Apaf-1 expression plasmid and differentiated before being treated with TNFα. The over-expression of Apaf-1 in IκBα-SR myotubes did not rescue sensitivity to TNFα-induced apoptosis, as measured by levels of cleaved caspase-3 following treatment. Collectively, these findings indicate that the transcription factor NF-κB is vital to the survival of skeletal myoblasts, but not required to inhibit TNFα-induced apoptosis in skeletal myotubes. Additionally, we show that the reduction of Apaf-1 along the myogenic program is not required for the survival of terminally differentiated skeletal myotubes treated with TNFα. Future elucidation of the role of NF-κB and important anti-apoptotic mechanisms in mature skeletal muscle could lead to a better understanding of skeletal muscle differentiation as well as novel insight into numerous skeletal muscle diseases, including the pediatric muscle cancer rhabdomyosarcoma.

VITA

2002-2006……..**Oak Hills High School**
Cincinnati, Ohio

2006-2010……....**The Ohio State University**
Columbus, Ohio
Bachelor's of Science in Biomedical Science
Minor in Economics

2010……...**Northwestern University**
Evanston, Illinois
Pursuing a Doctorate of Philosophy in Biological Science

TABLE OF CONTENTS

CHAPTER 1 – INTRODUCTION

1.1 - Problem Statement and Significance

Skeletal muscle cell differentiation is the process by which mononucleated, proliferative myoblasts fuse to form multinucleated, contractually-competent muscle cells called myotubes [10]. *In vivo* this differentiation process is completed around the time of birth, and myotube regeneration can be a very arduous process [11]. Because of the strenuous demand of regeneration, it is an advantage for the organism to maintain a stable number of skeletal muscle cells. In line with this idea, mature skeletal muscle is observed as a very death-resistant tissue [12]. However, currently very little is known about the molecular mechanisms that account for this "hardy" phenotype.

One suggested mechanism for this hardiness is through differentiation's regulation of apoptotic peptidase activating factor-1 (Apaf-1). Apaf-1 is a pro-apoptotic protein located in the cytoplasm and found to be integral to most forms of apoptosis through its role in the apoptosome, a cytoplasmic protein complex composed of Apaf-1, cytochrome *C*, and procaspase-9 [13]. During skeletal muscle differentiation, the levels of Apaf-1 decrease significantly and are thought to greatly impair the myotubes ability to undergo programmed cell death as induced by the protein cytochrome *C* [9]. However, cytochrome *C* is not a readily occurring stimulus for apoptosis. It is located in the matrix of the mitochondria and used in the electron transport chain to produce ATP; it must be induced by another stimulus or stress signal to be released into the cytoplasm [13].

The inflammatory cytokine tumor necrosis factor alpha (TNFα) is one such stimulus [14]. Interestingly, TNFα has also been shown to be a key mediator in skeletal myopathies such as cachexia and muscular dystrophy [3, 5]. The cellular protein through

which TNFα exerts its deleterious effect in these diseases is NF-κB, a potent inhibitor of both differentiation [1-4] and apoptosis [6-8]. The response of skeletal muscle cells to TNFα during the differentiation process and the role of NF-κB in myotube survival could not only provide new information about skeletal muscle development and physiology, but it could also lead to new insight in the mechanisms of many skeletal myopathies.

1.2 - Background Information

NF-κB is a transcription factor from the Rel family of transcription factors. Currently, there are five known mammalian Rel proteins: RelA (p65), c-Rel, RelB, p50 and p52. These proteins are subunits of NF-κB and exist in cells as homodimers or heterodimers. The p65/p50 heterodimer is the NF-κB form shown to inhibit differentiation as well as apoptosis and will from here on be referred to as "NF-κB" [15].

In unstimulated cells, NF-κB is inactive and bound to an IκB family protein, specifically IκBα. IκBα traps the transcription factor in the cytoplasm by blocking important phosphorylation sites and the nuclear localization signal of the dimer. Because of this, the IκB protein must be degraded before NF-κB can be activated and translocate to the nucleus [15]. To degrade, IκBα must first be phosphorylated at serine residues 32 and 36. This phosphorylation tags the protein for polyubiquitination and, ultimately, degradation by the proteasome [15].

NF-κB is activated by many signals, including hypoxic conditions, ionizing radiation, cell stress, and inflammatory cytokines [15]. Once activated, NF-κB enters the nucleus and promotes the transcription of a vast assortment of genes involved in

numerous of biological processes, including skeletal muscle differentiation and apoptotic regulation [15].

Studies have reported several mechanisms by which NF-κB is an inhibitor of apoptosis [6-8, 16]. Apoptosis, or programmed-cell death, is characterized by membrane blebbing, chromatin condensation, nuclear fragmentation, and an overall reduction in cellular volume [17]. Mechanistically, apoptosis occurs as a result of intrinsic or extrinsic stimuli that trigger the systematic production and activation of caspases, proteases that dismantle the cell through the cleavage of intracellular substrates [17].

One such extrinsic apoptotic signal is the inflammatory cytokine TNFα. To signal apoptosis, TNFα binds to and causes the trimerization of TNF receptor-1 (TNFR1) [14]. TNFR1 trimerization signals the eventual formation of the death-inducing signaling complex (DISC), also know as complex II. This cytoplasmic complex consists of TNF-receptor associated factor 2 (TRAF2), TNF-receptor associated death domain (TRADD), Fas-associated death domain (FADD), the kinase RIP1, and the progenitor forms of caspases-8 and -10 [18]. The formation of DISC results in the cleavage of procaspases-8 and -10 to their activated counterparts. Caspases-8 and -10 then cleave a protein called to Bid to a truncated form (tBid), which interacts with anti-apoptotic Bcl-2 family proteins and results in the release of cytochrome *C* from the mitochondrion [19-21]. Cytoplasmic cytochrome *C* binds with procaspase-9 and Apaf-1 in an ATP-dependent manner to form a molecular machine known as the apoptosome [19-21]. Once activated, caspase-9 cleaves the effector caspase, caspase-3. Caspase-3 then activates caspase-activated deoxyribonuclease (CAD) by cleavage of the enzyme's inhibitor domain, allowing CAD to enter the nucleus and degrade chromosomal DNA [19-21].

Paradoxically, an hour before the DISC complex forms, TNFα signals the formation of a different multi-protein complex. This complex, termed complex 1, forms directly on the activated, membrane-bound TNFR1 and signals the activation of NF-κB [18, 20-21]. Complex 1 is comprised of TNFR1, TRAF2, TRADD, and RIP1, the kinase believed to be responsible for the TNFα-dependent stimulation of the inhibitor of κB kinase (IKK) complex [18]. The IKK complex then phosphorylates IκBα leading to its degradation and the subsequent activation of NF-κB [18]. NF-κB activation leads to an increase in the transcription of NF-κB regulated genes, including the anti-apoptotic genes, *cIAP1, cIAP2, Bcl-xL, A1,* and *XIAP* [20-21]. These genes transcribe proteins that inhibit the TNFα-induced apoptotic cascade at various points (Appendix A.1). Because complex 1 forms an hour before the DISC complex and up-regulates the transcription of anti-apoptotic genes through NF-κB, TNFα alone does not kill most healthy, wild-type cells. However, in cells lacking competent NF-κB activity, TNFα is a potent apoptotic agent [6-8].

In skeletal muscle cells, NF-κB also plays a role in regulating differentiation. Differentiation is the process through which a cell transforms from less tissue specific to more tissue specific; this transformation is characterized by an exit from the mitotic cell cycle [22]. NF-κB inhibits this differentiation process by trapping the cell in the replicative cycle [1-2] and inhibiting the production of muscle-specific genes, through both transcriptional repression and post-transcriptional modification [3-4].

1.3 - Objectives

It was previously reported that NF-κB is an important molecular mediator of muscle cachexia [3] and an inhibitor of skeletal muscle differentiation [1-4]. However, in one study [3], it was observed that C2C12 myotubes lacking NF-κB activity (via a stable infection of a dominant negative IκBα mutant IκBα-SR) do not exhibit morphological signs of cell death following TNFα treatment.

Our objective for this study was to investigate the role of skeletal muscle differentiation on the molecular machinery responsible for carrying out TNFα-induced apoptosis. Particular interest was taken in NF-κB independent mechanisms of apoptotic inhibition, including the proposed down-regulation of the pro-apoptotic protein Apaf-1 that occurs during differentiation [9].

Confirm the lack of TNFα-induced apoptosis in C2C12 IκBα-SR myotubes.

The first aim of this study was to replicate previous findings [3] that C2C12 myotubes did not require NF-κB to inhibit TNFα-induced apoptosis. Additionally, we sought to confirm that NF-κB was vital to C2C12 myoblast survival against TNFα stimulation and that TNFα treatment induced apoptosis in the absence of functional NF-κB signaling.

Confirm the presence of a fully functional TNFα signaling pathway in C2C12 myotubes.

Next, we wanted to confirm that differentiated C2C12 cells were able to functionally sense the inflammatory cytokine TNFα. If the cytokine was not properly

received by TNFR1, then the absence of apoptosis in IκBα-SR myotubes could simply be due to the differentiated cell's insensitivity to TNFα.

Investigate the effects of differentiation and NF-κB inhibition on the pro-apoptotic protein Apaf-1.

It was recently proposed that skeletal muscle differentiation evokes a natural reduction of Apaf-1 [9], but as of yet, very little is known about anti-apoptotic mechanism. By investigating this phenomenon in the absence of the NF-κB, the possible role of this transcription factor in Apaf-1 reduction can be examined. If NF-κB appeared to play no direct role in the event, it could be hypothesized that the natural reduction of Apaf-1 during differentiation was at least partially responsible for the NF-κB independent inhibition of apoptosis seen in skeletal myotubes.

Determine the role of the Apaf-1 in TNFα-induced myotube apoptosis.

Finally, we sought to better determine the role of Apaf-1 in TNFα-induced apoptosis in C2C12 myotubes by transfecting C2C12 Vector and IκBα-SR cells with an Apaf-1 expression plasmid. Once these cells were transfected to over-express Apaf-1, they could be differentiated to form myotubes, treated with TNFα, and examined for markers of apoptosis.

CHAPTER 2 - MATERIALS AND METHODS

Cell Culture

Murine C2C12 skeletal myoblasts were cultured in 5% CO_2 at 37°C in Dulbecco's modified Eagle's medium (DMEM), supplemented with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin. The cells were grown below confluency and passaged every 3 to 4 days. To induce differentiation, the cells were grown overnight to 50 to 60% confluency in growth medium (GM), washed once with phosphate-buffered saline (PBS), and then switched to DMEM supplemented with 2% horse serum and 10 mg of insulin per ml plus antibiotics (differentiation medium or DM). Stable cell lines expressing the mutant dominant negative IκBα protein (IκBα-SR) and the control vector cell line were prepared as previously described [1]. All media and other cell culture reagents were purchased from GIBCO-Invitrogen (California, United States).

Nuclear Extracts

Cells grown in 100 mm diameter dishes were washed twice with phosphate-buffered saline, gently scraped from the plates, transferred to microcentrifuge tubes, and lysed on ice in cytoplasmic extraction buffer (10 mM HEPES [pH 7.6], 60 mM KCl, 1 mM EDTA, 0.1% Nonidet P-40). Nuclei were pelleted (2600 rpm, 4°C, 4 min). Nuclei were washed gently with 100 ml of cytoplasmic extraction buffer without Nonidet P-40 and pelleted (2600 rpm, 4°C, 4 min). Nuclear extraction buffer (20 mM Tris [pH 8.0], 420 mM NaCl, 1.5 mM MgCl2, 0.2 mM EDTA, 0.5 mM phenylmethylsulfonyl fluoride, 25% glycerol) was added to the pellets. Nuclear pellets were then resuspended by vortexing, and the nuclear lysates were maintained on ice for 10 min with additional periodic

vortexing. Nuclear extracts were cleared (13,200 rpm, 4°C, 10 min) and transferred to fresh tubes. Protein concentrations were determined by optical densitometry using the Bradford assay and all extracts were stored at -80°C.

Electrophoretic Mobility Shift Assay

5 μg of nuclear extract was pre-incubated with 50 mM phenylmethylsulfonyl fluoride and 1 μg poly(dI-dC) (Amersham Biosciences) for 10 minutes at room temperature. This mixture was then incubated with 20,000 cpm of a ^{32}P-labeled oligonucleotide probe containing an NF-κB binding site in a total volume of 20 μl binding buffer for 20 minutes at room temperature. The binding buffer was made up of 50 mM Tris-HCl (pH 7.6), 2.5 mM EDTA, 5 mM dithiothreitol, and 50% glycerol. Protein-oligonucleotide complexes were resolved on a 5% acrylamide gel in TGE buffer (25 mM Tris, 190mM glycine, and 1 mM EDTA) at 25 mA for 2.5 hours. The gel was dried and exposed on a phosphoscreen overnight before being scanned on a Typhoon scanner (Amersham Biosciences).

Luciferase Assay

Cells were grown to 50% confluency in 12 well plates and cotransfected with 0.250 μg 3xκB-Luc [1], a reporter plasmid containing 3 NF-κB binding sites in front of the luciferase gene, and 0.250 μg CMV-LacZ, a reporter plasmid used to monitor transfection efficiency, using Lipofectamine reagent (Invitrogen). Plasmids were incubated in Opti-Mem medium with Lipofectamine and Plus Reagent (Invitrogen) for 30 minutes before being added to cells in 1 ml Opti-Mem. Cells were incubated in transfection medium for

2 to 3 hours, washed in PBS, and allowed to grow overnight in GM. The cells were then treated, washed in PBS, and collected using M-PER reagent (Pierce). Luciferase assays were performed using 40 µl of cell lysate in a white 96-well plate with 1 mM luciferin substrate on a Veritas Luminometer. Transfection efficiency was measure by β-galactodiose assay with optical densitometry using ONPG as a substrate.

Western Blot Analysis

Cells were harvested in PBS, and whole-cell lysates were prepared by suspending cell pellets in ice-cold RIPA buffer (10 mM Tris-HCl [pH 8.0], 1 mM EDTA, 1% sodium dodecyl sulfate [SDS], 1% Nonidet P-40) and incubating the suspension on ice for 10 min. For Western blots of phosphorylated proteins, lysis buffer included phosphatase inhibitors (50 mM sodium fluoride, 1 mM orthovanadate, 50 mM b-glycerophosphate, 80 mM cantharidin). Supernatant lysates were collected following high-speed centrifugation (5,000 rpm) for 5 min at 4°C. Equal amounts of protein extract, as determined by protein optical density measurement via the Bradford assay, were subjected to SDS-polyacrylamide gel electrophoresis and transferred to methanol-activated polyvinylidene fluoride membranes (Millipore). Blocking was performed in 5% nonfat dry milk TBST (25 mM Tris-HCl [pH 8.0], 125 mM NaCl, 0.1% Tween 20). Primary antibodies were diluted in 3% bovine serum albumin TBST and membranes incubated at 4^{o}C overnight. Secondary antibodies were diluted in 0.5% nonfat dry milk TBST and incubated for 1 hour at room temperature. Washes were performed in TBST for 5 to 10 min and repeated three times between incubations. Specific protein bands were visualized by enhanced chemiluminescence (Perkin Elmer). Antibodies to cleaved caspase-3, phospho-p38, p38,

and myogenin were obtained from Santa Cruz Biotechnology (California, United States), Apaf-1 antibody obtained from Alexis Biochemicals (California, United States), and α-tubulin antibody was procured from Sigma Aldrich (Missouri, United States). Secondary mouse and rabbit antibodies were obtained form Promega (Wisconsin, United States); secondary rat antibody was obtained from Alexis Biochemicals (California, United States).

Cell Counting and Phase Microscopy

Cells were cultured in 12-well plates and grown to 60-70% confluency following previously mentioned guidelines. Cells were photographed on an Olympus CK40 phase contrast microscope using a Nikon Coolpix 4500 with special microscope lens attachment. Following photographic documentation, cells were washed in PBS and incubated in 0.25% trypsin with EDTA (GIBCO-Invitrogen) for 4 minutes to remove cells from plate. Trypsinized cells were then resuspended in GM. Approximately 10 µl of cell suspension was then loaded into each side of a hemocytometer and cells were counted using previously listed phase contrast microscope. Cell suspension count was then multiplied by dilution factor to get the final cell count.

TUNEL Assay and Immunofluorescent Staining

All terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) experiments were performed directly in 35 mm dishes using the "In Situ Cell Death Detection Kit, Fluorescein" from Roche Applied Sciences (Indiana, United States). Myoblasts and myotubes were cultured then treated with TNFα from Roche at the listed concentrations

and time periods. Media was removed and cells were washed with PBS. Cells were then fixed with 4% paraformaldehyde for 1 hour at room temperature then rewashed with PBS. Cells were incubated in permeabilisation solution (0.1% Triton X-100 in 0.1% sodium citrate) for two minutes on ice. After a PBS wash and once dry, the sample was treated with TUNEL reaction mixture for 1 hour at 37^{o}C in a dark, humid incubator. The cells were washed 3x with PBS and permeabilized in 0.5% NP-40 for 5 minutes. Samples were washed twice with PBS and blocked for 30 minutes in horse serum (1:100 in PBS). Following two more PBS washes, the cells were incubated with myosin-heavy chain antibody (Santa Cruz, 1:500) in 3% BSA for 1 hour. Cells were then washed three times in PBS and incubated with secondary antibody (Molecular Probes, 1:250 in 3%BSA) for one hour. Samples were washed with PBS and dionized water, then coverslipped. Samples were examined by fluorescence microscopy.

Transient Transfection

Cells were grown to 50% confluency in 60 mm dishes transfected with 2.5 μg of either a plasmid containing the Apaf-1 gene linked to a CMV promoter (Invitrogen) or an empty CMV-linked pCDNA3 vector plasmid. Plasmids were allowed to incubate in 0.5 ml Opti-Mem medium with Lipofectamine and Plus Reagent (Invitrogen) for 30 minutes before being added to cells in 2 ml Opti-Mem. Cells were incubated in transfection medium for 2 to 3 hours, washed in PBS, and allowed to grow overnight in GM. Following overnight incubation, cells were washed and switched to DM for 72 hours before being collected.

Reverse Transcriptase-PCR

RNA was isolated with Trizol Reagent per the manufacturer's recommendations (Life Technologies). 5 μg of RNA isolated from cells under differing treatment conditions was converted to cDNA using M-MLV reverse transcriptase (Invitrogen) and recommendations of the manufacturers. cDNA mixture was then diluted with 80 μl DEPC-H2O and 2 μl was used as a template in a PCR with Taq DNA polymerase (New England Biolabs) using the following primer pairs:

cIAP: Forward – 5'-GCTTCTACTACATAGGACCTGG-3'
Reverse – 5'-CACTTGACATCATCACTGTGTCC-3'

GAPDH: Forward – 5'-CAGGGCAAATTCAACGGCACAGTCAAGG-3'
Reverse – 5'-GTTCACACCCATCACAAACATGG-3'

PCR mixture samples were run on an ethidyum-bromide spiked agarose gel and viewed using an AlphaImager (Alpha Innotech).

CHAPTER 3 - RESULTS

3.1 - Inhibition of NF-κB in C2C12 cell line via IκBα-SR expression

The stable C2C12 murine myoblast cell lines used for this investigation, one expressing a dominant negative form of IκBα called IκBα-super repressor (IκBα-SR) and the other an empty vector plasmid (Vector), were previously created by retroviral infection [1]. This dominant negative form of IκBα cannot be phosphorylated properly due to mutations at serine residues 32 and 36 [15]. The loss of these phosphorylation sites on IκBα inhibits activation of NF-κB by preventing ubiquitin-mediated degradation by the proteasome. Without the IκBα degradation, NF-κB is trapped in the cytoplasm and unable to regulate transcription (Diagram A.2).

To verify the inhibition of NF-κB in the C2C12 IκBα-SR cell line, two experiments were performed (Figure B.1). First, to show the cytoplasmic trapping of NF-κB, a gel shift was performed (Figure B.1 - A). C2C12 Vector myoblasts exhibited basal levels of activated NF-κB as well as increased levels following both 15 and 30 minutes of TNFα treatment. Both treated and untreated C2C12 IκBα-SR myoblasts exhibited no levels of activated, nuclear NF-κB.

Functional inhibition of NF-κB was verified by luciferase assay using transient transfection of the 3xκB-Luc plasmid [1] (Figure B.1 – B). C2C12 Vector myoblasts exhibited a TNFα dependent increase in luciferase activity, while C2C12 IκBα-SR myoblasts had only negligible levels of luciferase activity and were unaffected by TNFα treatment. This absence of TNFα induced luciferase activity indicates that C2C12 IκBα-SR cells have a loss of NF-κB dependent transcriptional activity.

3.2 - IκBα-SR myoblasts are sensitive to TNFα-induced apoptosis

In previous studies [3], myotubes expressing the mutant IκBα-SR protein have been treated with TNFα to show that NF-κB positively regulates cachexia. While such experiments have shown that myotubes devoid of NF-κB activity do not undergo cachectic degradation, the experiments have also shown that such myotubes do not undergo the expected TNFα-induced apoptosis [3]. In accordance with this theory, it was hypothesized that inhibition of NF-κB in myoblasts would sensitize the cells to TNFα-induced apoptosis, while the inhibition of NF-κB in myotubes would not sensitize the cells to TNFα-induced apoptosis. To test this hypothesis, C2C12 IκBα-SR cells were treated with TNFα during various periods of skeletal muscle differentiation.

As expected, undifferentiated IκBα-SR myoblasts saw a significant increase in cell death following TNFα treatment as seen by an increase in floating cells. (Figure B.2 - A). IκBα-SR myoblast death was quantified following 24 hours of TNFα treatment (Figure B.2 - B), and a viability decrease of approximately 80% was seen in IκBα-SR myoblasts treated with TNFα concentrations between 5 ng/ml to 20 ng/ml. A smaller decrease in repressor myoblast viability was seen using TNFα concentrations less than 5 ng/ml (data not shown) indicating that 5 ng/ml was the minimum TNFα concentration required to get the maximum response.

To confirm that the increase in C2C12 IκBα-SR myoblast cell death elicited by TNFα treatment was in fact due to apoptosis, both a TUNEL assay and a Western blot for cleaved caspase-3 were performed. The TUNEL assay is a chemical method for detecting DNA fragmentation, a characteristic trait of cell death. IκBα-SR myoblasts treated with TNFα (5 ng/ml for 4 hours) showed TUNEL positive nuclei, as evidenced by

the colocalization of TUNEL and DAPI staining. C2C12 Vector cells showed no positive TUNEL staining following TNFα treatment (Figure B.2 - C).

Whole cell extracts of TNFα-treated Vector and IκBα-SR myoblasts were analyzed by Western blot. Treated and untreated C2C12 Vector myoblasts presented with minimal levels of the apoptotic marker cleaved caspase-3, confirming the absence of cell death seen by other measures. C2C12 IκBα-SR myoblasts exhibited a TNFα (5 ng/ml for 4 hours) dependent increase in cleaved caspase-3 levels, consistent with what would be found in cells experiencing TNFα-induced apoptotic cell death [8, 17, 19] (Figure B.2 – D).

3.3 - IκBα-SR myotubes are insensitive to TNFα-induced apoptosis.

Unlike their undifferentiated counterparts, C2C12 IκBα-SR myotubes exhibited no decrease in cell viability following TNFα treatment (Figure B.3). Under phase microscopy, IκBα-SR myotubes exhibited no morphological changes following TNFα treatment of up to 20 ng/ml for 24 hours (Figure B.3 – A). When quantified by cell count, there was no statistically significant change in cell viability; the slight decrease in IκBα-SR myotube viability was believed to be due to death from cells unable to differentiate *in vitro*. TUNEL assays and cleaved caspase-3 westerns were carried out using C2C12 myotubes and results were negative for both Vector and IκBα-SR cell lines (Figure B.3 – C, D).

3.4 - Skeletal myotubes possess a functional TNFα signaling pathway.

To test the sensitivity of transfected C2C12 myotubes to TNFα, Western blot analysis was performed on protein lysates from TNFα-treated Vector and IκBα-SR myotubes. The proteins of interest in this experiment were the phosphorylated forms of the mitogen-activated protein kinases (MAPKs) p38 and JNK. The p38 and JNK signaling pathways are two intracellular cascades that are activated by TNFα via an NF-κB independent manner [14]. In both Vector and IκBα-SR myotubes, TNFα treatment (5 ng/ml) signals the activation of the p38 and JNK pathways as visualized by phosphorylated forms of the MAPKs (Figure B.4 - A). The activation of these two signaling cascades by TNFα confirms that transfected C2C12 myotubes are sensitive to the inflammatory cytokine and that the absence of apoptosis in TNFα-treated IκBα-SR myotubes (Figure B.3) was not simply due to an absence of activation by TNFα.

TNFR1 sensitivity was confirmed functionally in differentiated C2C12 skeletal muscle cells using reverse transcriptase-polymerase chain reaction (RT-PCR). Vector and IκBα-SR myotubes were treated with TNFα (5 ng/ml) for periods of 0, 2, and 4 hours. Total RNA was then collected from these cells, complimentary DNA (cDNA) was formed by reverse transcription, and PCR was carried out probing for cIAP transcript. cIAP is a NF-κB regulated anti-apoptotic protein [16, 20-21] and as a result, it can be seen to be up-regulated specifically in Vector myotubes following 4 hours of stimulation with TNFα. The absence of cIAP up-regulation in IκBα-SR myotubes further confirms the inhibition of NF-κB in these cells (Figure B.4 – B).

3.5 - Skeletal muscle differentiation evokes a natural reduction of Apaf-1

Next, we set out to monitor the changes in Apaf-1 levels during differentiation. Using whole cell lysates of both C2C12 cell lines collected during the differentiation process (0, 24, 48, 72 hours DM), Western blot analysis probing for Apaf-1 was performed. As shown previously [9], Apaf-1 levels decreased during the differentiation process and were almost completely ablated in terminally differentiated Vector myotubes (Figure B.5). The differentiation marker myogenin was used to show that decreasing Apaf-1 levels corresponded with progression along the skeletal muscle differentiation program. Interestingly, it was observed that this natural reduction of Apaf-1 was not dependent on NF-κB as the same reduction was seen in IκBα-SR cells (Figure B.5).

3.6 - Over-expression of Apaf-1 does not rescue apoptosis in TNFα treated IκBα-SR myotubes.

To test whether the reduction of the pro-apoptotic protein Apaf-1 was responsible for the NF-κB independent inhibition of apoptosis seen in IκBα-SR myotubes, the Apaf-1 levels in these cells were restored using an expression plasmid and the cells were then treated with TNFα to see if apoptosis was rescued. To restore Apaf-1 levels, IκBα-SR myoblasts were first transiently transfected with an Apaf-1 expression plasmid. Then following transfection, the cells were allowed to differentiate for 72 hours. Following differentiation, Apaf-1 over-expressing IκBα-SR myotubes were treated with TNFα (5 ng/ml) for 4 hours. Whole cell lysates from these cells were analyzed by Western blot analysis for cleaved caspase-3. Unlike the previous study where the restoration of Apaf-1 levels sensitized myotubes to cytochrome *C* induced apoptosis [9], the transiently

transfected Apaf-1 over-expressing IκBα-SR myotubes were not sensitized to TNFα-induced apoptosis when compared with control pCDNA3 transfected IκBα-SR myotubes (Figure B.6).

CHAPTER 4 – DISCUSSON

Using C2C12 myoblasts as an *in vitro* model of skeletal muscle, the data show that myotubes devoid of NF-κB activity (via stable expression of IκBα-SR) were equally resistant to TNFα-induced apoptosis as Vector myotubes (Figure B.3). Furthermore, this NF-κB independent anti-apoptotic activity must arise during the differentiation process because myoblasts lacking NF-κB activity showed a significant sensitivity to TNFα through a substantial increase in cell death (Figure B.2). Vector myoblasts experienced no decrease in cell viability, verifying that the transfection process was not responsible for the increased TNFα responsiveness of IκBα-SR myoblasts (Figure B.2).

This increase in IκBα-SR myoblast cell death was due to apoptosis as signaled by TNFα, an inflammatory cytokine and known apoptotic factor [14]. Apoptosis was confirmed as the process responsible for the decrease in repressor myoblast viability via TUNEL assay and Western blot probing for the apoptotic marker cleaved caspase-3 (Figure B.2 – C, D).

The results further suggest that a down-regulation of TNFα signaling triggered by the onset of differentiation was not responsible for NF-κB independent mechanism of apoptotic resistance. The phosphorylation of both p38 and JNK as well as the up-regulation of cIAP transcription following TNFα treatment shows that C2C12 myotubes do receive the TNFα signal (Figure B.4). The MAPK activation also suggests that C2C12 IκBα-SR myotubes exhibit fully functional TNF signaling pathways and that some cellular process independent of NF-κB must be inhibiting the apoptotic signal of TNFα.

A recent publication claims that skeletal muscle differentiation down-regulates the expression of Apaf-1, a component of the apoptosome complex responsible for caspase-9 activation, and prevents the post-mitotic cell from undergoing cytochrome *C*-induced apoptosis [9]. Here we confirm that Apaf-1 levels are reduced during skeletal muscle differentiation and that this process occurs independently of NF-κB (Figure B.5). However, the over-expression of Apaf-1 in IκBα-SR myotubes was not enough to rescue sensitivity to TNFα-induced apoptosis (Figure B.6).

Future studies have been proposed to investigate the expression levels of many anti- and pro-apoptotic proteins during the differentiation program. It is hypothesized that skeletal muscle differentiation evokes changes in the expression levels of specific apoptotically relevant proteins in a way that promotes the survival of a myotube. Currently, we are beginning to investigate the Bcl-2 family of proteins. The Bcl-2 family is a collection of proteins that regulate apoptosis through their control of mitochondrial outer membrane permeabilization and subsequent cytochrome *C* release [23]. This family contains both anti- (Bcl-2, Bcl-xL, Mcl-w, Bcl-w, A1) and pro-apoptotic (Bax, Bak) members that interact in equilibrium to decide the fate of a cell [23]. When this equilibrium is disturbed and the pro-apoptotic proteins outnumber the anti-apoptotic ones, a stressful stimulus like TNFα can trigger pores to form in the outer membrane of the mitochondria, allowing cytochrome *C* to enter the cytoplasm and bind with Apaf-1 to form the apoptosome [13]. A more in-depth examination of the Bcl-2 family could help elucidate a new pro-survival mechanism found in skeletal myotubes and possibly explain why Apaf-1 over-expressing IκBα-SR myotubes are insensitive to TNFα-induced apoptosis.

Although incomplete, the results here support the notion that skeletal muscle differentiation evokes the manifestation of an NF-κB independent mechanism of apoptotic resistance to TNFα treatment. While there is currently no mechanism known to be responsible for this observation, it has been shown that the natural reduction of Apaf-1 that occurs during the differentiation program does not confer this ability to the myotube. Studies are being undertaken to examine the possible role of other apoptotically relevant proteins with the hope that a mechanism responsible for this phenomenon be discovered in the very near future. Once complete, this mechanism would not only provide new insight into the cellular physiology of differentiated skeletal muscle, but could also lead to a new understanding of the role of NF-κB in rhabdomyosarcoma, a pediatric cancer characterized by tumors consisting of partially differentiated skeletal muscle cells [24-26].

APPENDIX

Appendix A – Diagrams

Diagram A.1 – TNFα Signaling Pathways

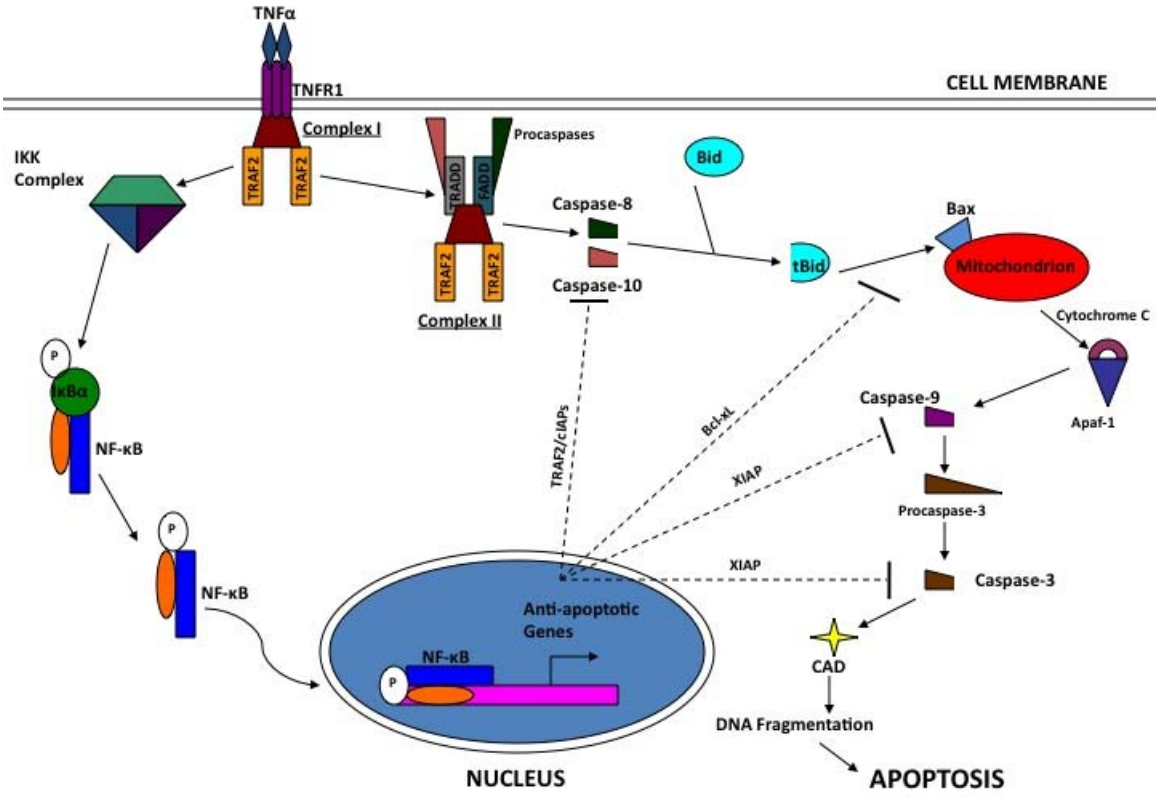

Diagram A.1 - TNFα-induced signaling. TNFα signals the apoptotic cascade through the cytoplasmic complex DISC (complex II), while also signaling the activation of the classical NF-κB pathway through membrane-bound complex I. NF-κB promotes the transcription of many anti-apoptotic genes which act to inhibit the apoptotic signal of TNFα through several mechanisms.

Diagram A.2 – Mechanism of NF-κB Inhibition by IκBα-SR

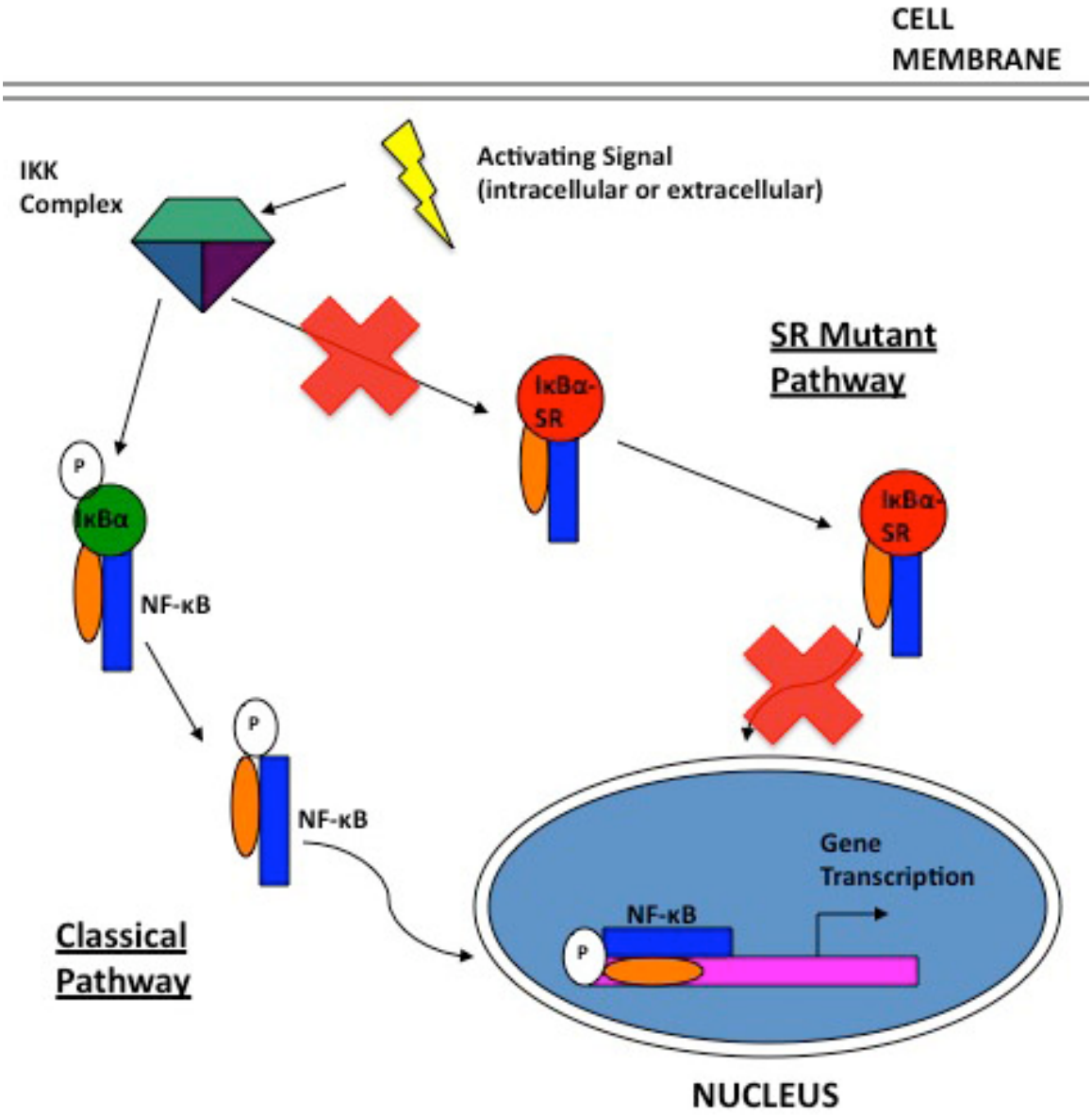

Diagram A.2 – Mechanism of NF-κB Inhibition by IκBα-SR. Expression of IκBα-SR inhibits NF-κB by trapping the transcription factor in the nucleus. Because the mutant IκBα protein cannot be phosphorylated, it can not be degraded and NF-κB cannot reach the nucleus. In the classical pathway, IκBα phosphorylation leads to proteasomal degradation of the protein and activation of NF-κB.

Appendix B – Figures

Figure B.1 – Inhibition of NF-κB in C2C12 cell line via IκBα-SR Expression

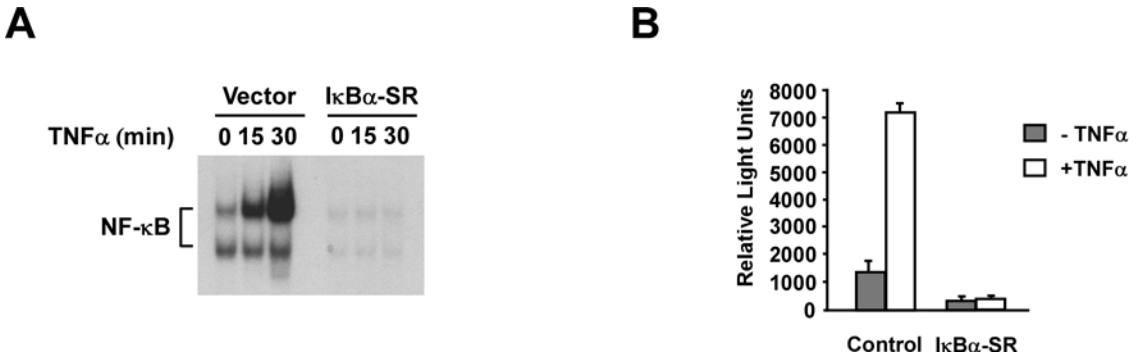

Figure B.1 - Inhibition of NF-κB in C2C12 cell line via IκBα-SR Expression. (A) Gel shift of NF-κB activation by TNFα. Nuclear extracts were collected from TNFα-treated C2C12 cells and a radioactive oligonucleotide probe was used to bind to activated nuclear NF-κB. (B) Luciferase reporter assay of NF-κB activity in Vector (control) and IκBα-SR myoblasts. Lysate of transfected cells were collected and luminescence was measured following incubation with luciferin substrate. Luminescence was normalized for transfection efficiency using β-galactodiose assay

Figure B.2 – IκBα-SR myoblasts are sensitive to TNFα-induced apoptosis.

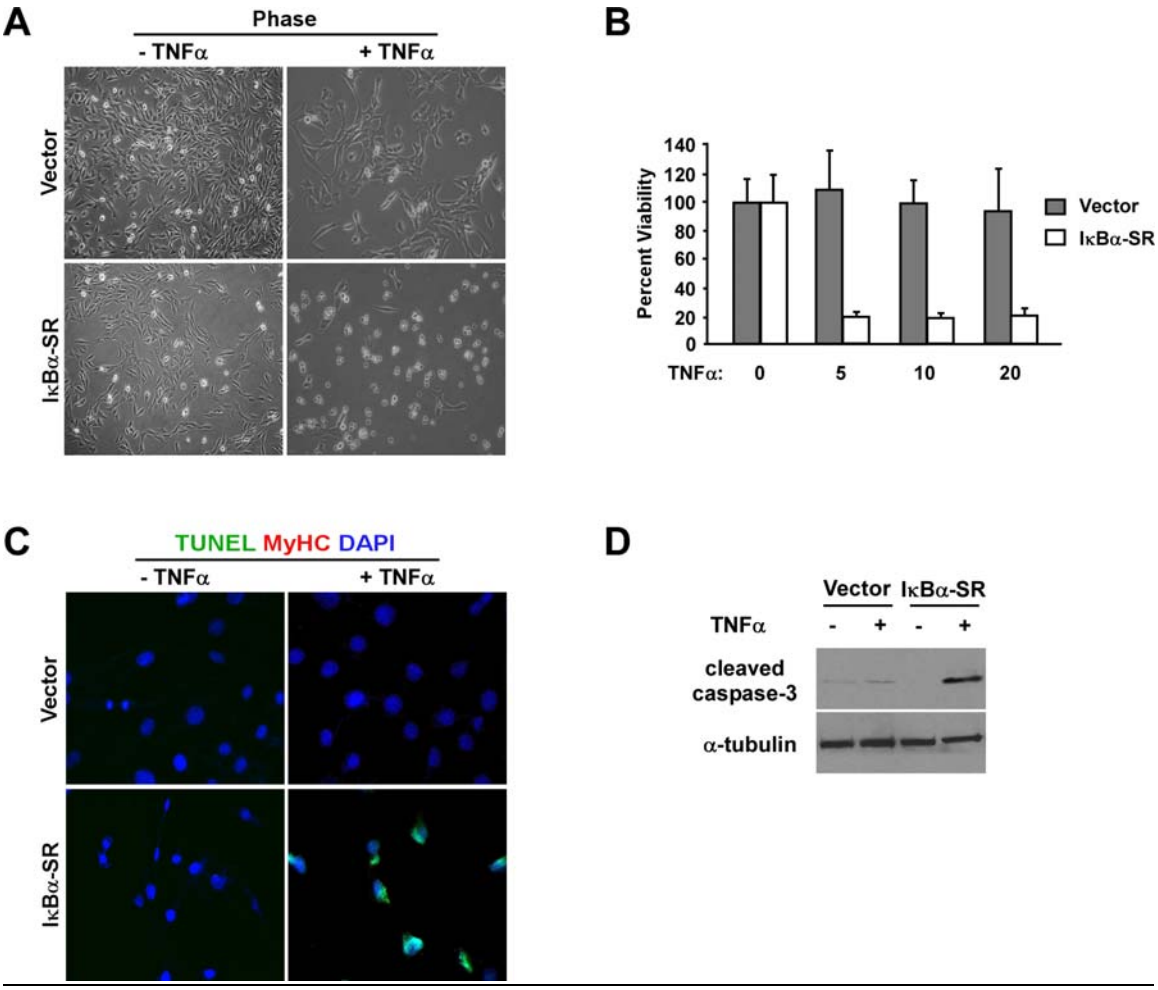

Figure B.2 – IκBα-SR myoblasts are sensitive to TNFα-induced apoptosis. (A) Cellular response to TNFα-treatment in Vector and IκBα-SR C2C12 myoblasts. Myoblasts were treated with TNFα (10 ng/ml) for 24 h; pictures were taken by phase microscopy. (B) TNFα-treated myoblast cell counts. Equal numbers of cells were plated, treated with TNFα for 24 hours, and counted using a hemocytometer. All counts normalized to untreated samples. (C) TUNEL Assay of Vector and IκBα-SR C2C12 myoblasts. Myoblasts were treated with TNFα (10 ng/ml) for 4 h; pictures were taken by fluorescence microscopy. (D) Western blot for cleaved caspase-3. Vector and IκBα-SR C2C12 myoblasts were treated with TNFα (5 ng/ml) for 4 h; cellular extracts were probed for cleaved caspase-3 by Western blot analysis.

Figure B.3 – IκBα-SR myotubes are insensitive to TNFα-induced apoptosis.

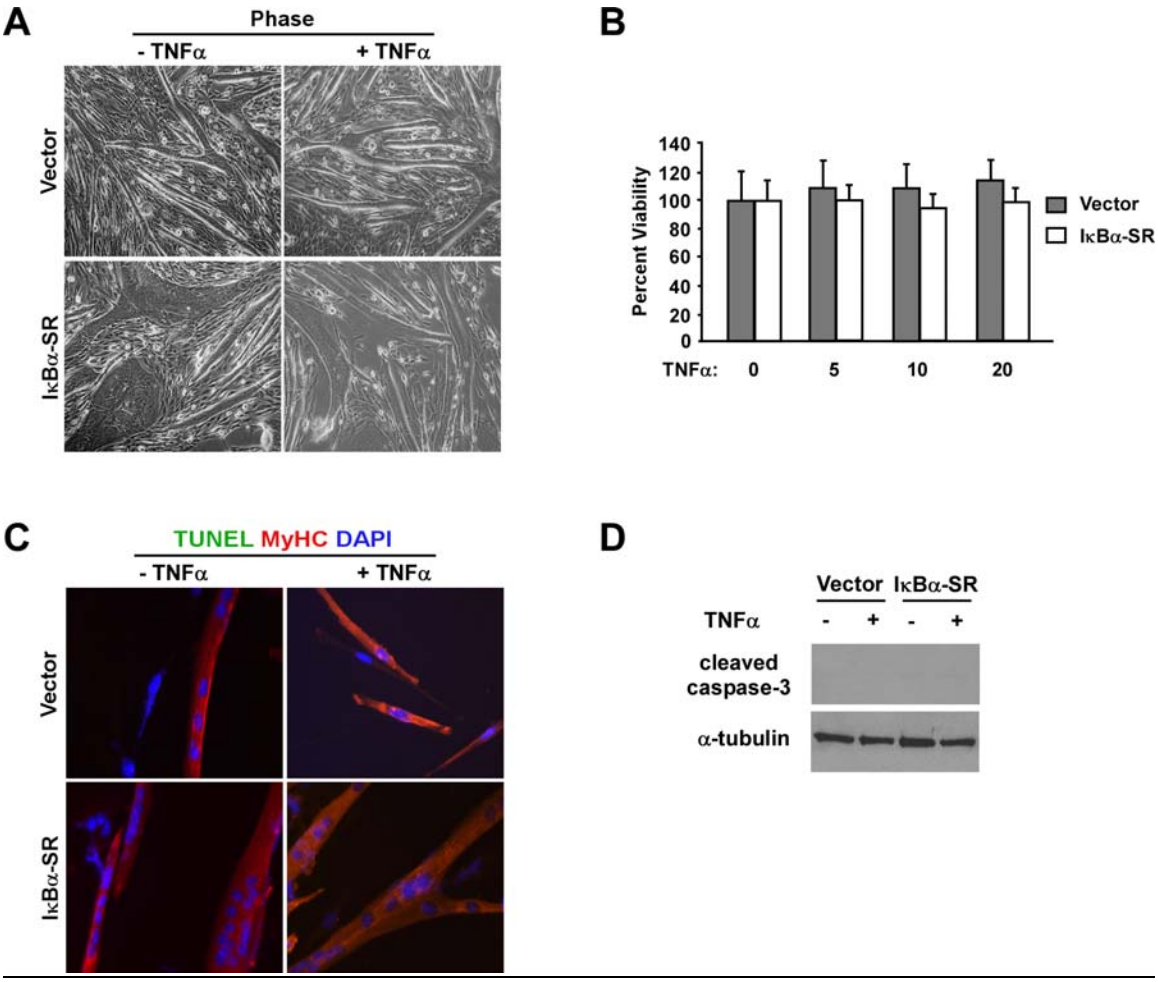

Figure B.3 – IκBα-SR myotubes are insensitive to TNFα-induced apoptosis. (A) Cellular response to TNFα-treatment in Vector and IκBα-SR C2C12 myotubes. Myotubes were treated with TNFα (10 ng/ml) for 24 h; pictures were taken by phase microscopy. (B) TNFα-treated myotube cell counts. Equal numbers of cells were plated and differentiated for 72 hours, then treated with TNFα for 24 hours, and counted using a hemocytometer. All counts normalized to untreated samples. (C) TUNEL Assay of Vector and IκBα-SR C2C12 myotubes. Myotubes were treated with TNFα (10 ng/ml) for 4 h; pictures were taken by fluorescence microscopy. (D) Western blot for cleaved caspase-3. Vector and IκBα-SR C2C12 myobubes were treated with TNFα (5 ng/ml) for 4 h; cellular extracts were probed for cleaved caspase-3 by Western blot analysis.

Figure B.4 – C2C12 myotubes possess a functional TNFα signaling pathway.

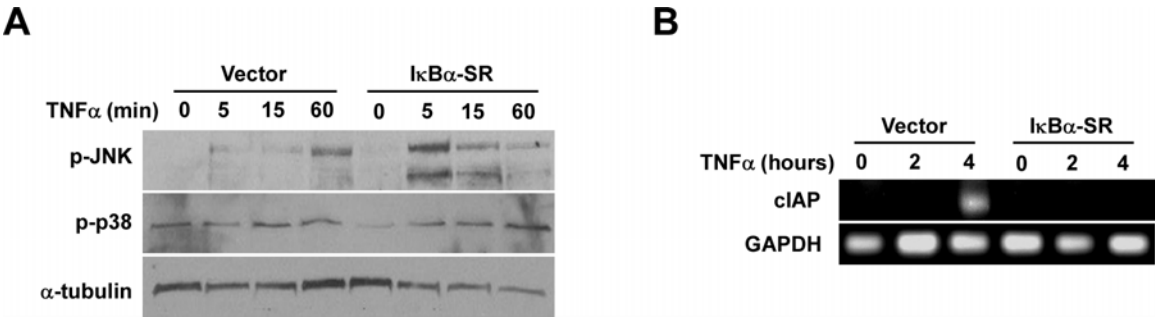

Figure B.4 – C2C12 myotubes possess a functional TNFα signaling pathway. (A) Western blot of proteins activated by TNFα treatment. Vector and IκBα-SR C2C12 myotubes were treated with TNFα (5 ng/ml) for varying amounts of time and cellular extracts were probed for phosphorylated JNK (p-JNK) and p38 (p-p38) by Western blot analysis. (B) Reverse transcriptase- polymerase chain reaction of TNFα-treated myotubes. Vector and IκBα-SR myotubes were treated with TNFα (5 ng/ml) for 2 and 4 h. RNA was then isolated and PCR was carried out probing for cIAP transcript.

Figure B.5 – Skeletal muscle differentiation evokes a natural reduction of Apaf-1.

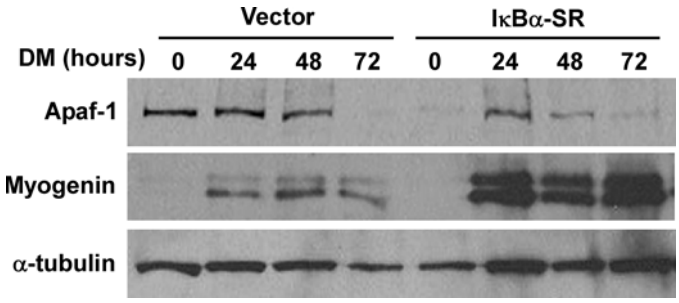

Figure B.5 – Skeletal muscle differentiation evokes a natural reduction of Apaf-1. Apaf-1 protein expression levels in myoblasts and myotubes. Vector and IκBα-SR C2C12 cells grown in growth medium then switched to differentiation medium and collected every 24 h until terminal differentiation at 72 h. Whole cell lysates from collected cells were probed for Apaf-1 and myogenin by Western blot analysis.

Figure B.6 – Over-expression of Apaf-1 does not rescue apoptosis in TNFα-treated IκBα-SR myotubes.

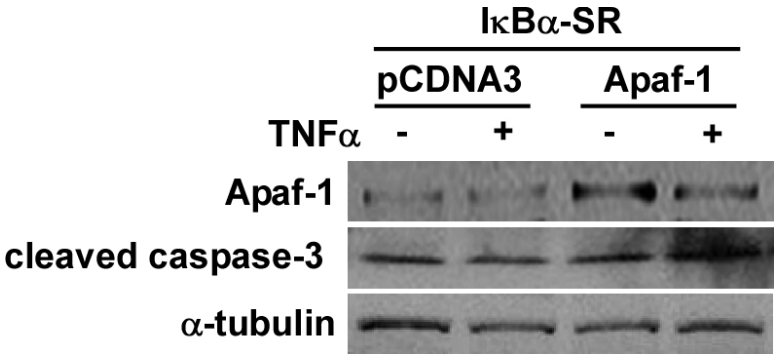

Figure B.6 – Over-expression of Apaf-1 does not rescue apoptosis in TNFα-treated IκBα-SR myotubes. C2C12 IκBα-SR myoblasts were transfected with either Apaf-1 expression plasmid or control pCDNA3 plasmid and allowed to differentiated for 72 h. Transfected IκBα-SR myotubes were then treated with TNFα (5 ng/ml) for 4 h; whole cell lysates were collected and protein levels were analyzed by Western blot.

REFERENCES

1. Guttridge, D. C., et al. 1999. NF-kappaB controls cell growth and differentiation through transcriptional regulation of cyclin D1. Mol Cell Biol 19:5785-5799.

2. Dahlman, J. M., et al. 2009. The RelA/p65 subunit of NF-kappaB specifically regulates cyclin D1 protein stability: implications for cell cycle withdrawl and skeletal myogenesis. J Cell Biochem 106:42-51

3. Guttridge, D. C., et al. 2000. NF-kappaB-Induced Loss of MyoD Messenger RNA: Possible Role in Muscle Decay and Cachexia. Science 289:2363-2366.

4. Wang, H., et al. 2007. NF-kappaB regulation of YY1 inhibits skeletal myogenesis through transcriptional silencing of myofibrillar genes. Mol Cell Biol 27:4374-4387.

5. Acharyya, S. et al. 2007. Interplay of IKK/NF-kappaB signaling in macrophages and myofibers promotes muscle degeneration in Duchenne muscular dystrophy. J Clin Invest 117:889-901.

6. Beg, A.A., and D. Baltimore. 1996. An Essential Role for NF-kappaB in Preventing TNFα-induced Cell Death. Science 274:782-784.

7. Van Antwerp, D.J., et al. 1996. Suppression of TNFα-induced Apoptosis by NF-kappaB. Science 274:787-789.

8. Wang, C-Y., et al. 1996. TNF- and Cancer Therapy-Induced Apoptosis: Potentiation by Inhibition of NF-kappaB. Science 274:784-787.

9. Smith, M. I., et al. 2009. Skeletal Muscle Differentiation Evokes Endogenous XIAP to Restrict the Apoptotic Pathway. PLoS ONE 4:e5097.

10. Widmaier, E. P., et al. 2006. *Vander's Human Physiology: The Mechanisms of Body Function, 10th Edition*. McGraw-Hill, New York.

11. Buckingham, M. et al. 2003. The formation of skeletal muscle: from somite to limb. J Anat 202:59-68.

12. Sullivan, J.D., et al. 1986. The properties of skeletal muscle. Orthop Rev 15:349-363.

13. Riedl, S. J. and G. S. Salvesen. 2007. The apoptosome: signaling platform of death. Nat Rev Mol Cell Biol 8:405-413.

14. Locksley, R. M., et al. 2001. The TNF and TNF Receptor Superfamilies: Integrating Mammalian Biology. Cell 104:487-501.

15. Ghosh, S., and M. Karin. 2002. Missing pieces in the NF-kappaB puzzle. Cell 109:S81-S96.

16. Wang, C-Y., et al. 1998. NF-kappaB Antiapoptosis: Induction of TRAF1 and TRAF2 and c-IAP1 and c-IAP2 to Suppress Caspase-8 Activation. Science 281:1680-1683.

17. Blank, M. and Y. Shiloh. 2007. Programs for Cell Death. Cell Cycle 6:686-695.

18. Micheau, O. and J. Tschopp. 2003. Induction of TNF Receptor 1-Mediated Apoptosis via Two Sequential Signaling Complexes. Cell 114:181-190.

19. Schultz, D.R., and W.J. Harrington Jr. 2003. Apoptosis: Programmed Cell Death at a Molecular Level. Semin Arthritis Rheum 32:345-369.

20. Dutta, J., et al. 2006. Current insights into the regulation of programmed cell death by NF-kappaB. Oncogene 25:6800-6816.

21. Kucharczak, J., et al. 2003. To be, or not to be: NF-kappaB is the answer – role of Rel/NF-kappaB in the regulation of apoptosis. Oncogene 22:8961-8982.

22. Gilbert, S. F. 2000. *Developmental Biology*, 6th Ed. Sinauer Associates, Inc; Sunderland, Massachusetts.

23. Leber, B., et al. 2007. Embedded together: The life and death consequences of interaction of the Bcl-2 family with membranes. Apoptosis 12:897-911.

24. Wang, H., et al. 2008. NF-kappaB – YY1 – miR-29 Regulatory Circuitry in Skeletal Myogenesis and Rhabdomyosarcoma. Cancer Cell 14:369-381.

25. Parham, D. M., and D. A. Ellison. 2006. Rhabdomyosarcomas in adults and children. Arch Pathol Lab Med 130:1454-1465.

26. Qualman, S.J., et al. 1998. Intergroup Rhabdomyosarcoma Study: update for pathologists. Pediatr Dev Pathol 1:550-61.